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COMPARATIVE BIOCHEMISTRY AND METABOLISM PART I: CARCINOGENESIS

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FOR THE COMMANDER

ROGER C. INMAN, Colonel, USAF, BSC
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O^6-methylguanine	N-nitrosopyrrolidine
monomethylhydrazine	methylation of liver DNA guanine

20. ABSTRACT *(Continue on reverse side if necessary and identify by block number)*

The inorganic reducing agent, hydrazine, is toxic and weakly carcinogenic. In earlier studies it was found that oral administration of a toxic dose of hydrazine to the rat resulted in methylation of liver DNA guanine moieties with an apparent dependency upon endogenous S-adenosylmethionine as the source of the methyl group. Further studies have been carried out to confirm the identity of these methylated bases in DNA as 7-methylguanine and O^6-methylguanine, to determine the rates of their formation and the duration of their persistence in DNA, and to investigate possible mechanisms by which hydrazine might stimulate the

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methylation of liver DNA.

The dose-response relationship for hydrazine and methylation of liver DNA were determined in the Fischer 344 rat and Syrian golden hamster. In both species the dose-response curves were essentially flat, except for the rat where the curve rose sharply at the approximate LD_{50}. The LD_{50} was not reached in the hamster at 90 mg/kg body weight; this species was found to tolerate 105 mg/kg and the LD_{50} is approximately 120 mg/kg body weight. Both 7-methylguanine and O^6-methylguanine form within minutes after oral administration of hydrazine to rats and hamsters. The kinetics of the removal of these two bases from liver DNA appears to be the same as described by other investigators for the removal of 7-methylguanine and O^6-methylguanine from rat and hamster liver DNA after administration of the strong carcinogen, dimethylnitrosamine. It has been proposed that S-adenosylmethionine may methylate hydrazine in the liver to monomethylhydrazine, which then could be metabolically activated to a methylating agent. Liver DNA from mice and hamsters contained considerably more 7-methylguanine and O^6-methylguanine following administration of hydrazine compared to administration of an equimolar dose of monomethylhydrazine, making the latter an unlikely intermediate in DNA methylation in hydrazine-treated animals. It was also proposed that S-adenosylmethionine could be a direct methylating agent and normally sequestered in the cell by the nucleophile, glutathione; a toxic dose of hydrazine then might deplete hepatic stores of glutathione and thus allow S-adenosylmethionine to methylate other cellular nucleophiles such as guanine in DNA. Depletion of hepatic glutathione levels with buthionine sulfoximine failed to alter S-adenosylmethionine levels or the methylation of liver DNA in rats subsequently poisoned with hydrazine. Glutathione, then, does not appear to have an important role in the DNA methylation response to hydrazine administration. The DNA methylation response did not occur in rats after given a dose of ethionine which resulted in depletion of S-adenosylmethionine levels return to normal, the methylation response was normal, in spite of a presumed high concentration of S-adenosylethionine, an inhibitor of most S-adenosylmethionine-dependent methyltransferases. These results suggest that such enzymes are probably not involved in liver DNA methylation following hydrazine administration. The identity of O^6-methylguanine in liver DNA of hydrazine-poisoned rats has been confirmed by Dr. Peter F. Swann of the Courtauld Institute of Biochemistry in London using a radioimmunoassay. The formation of 7-methylguanine has been detected in liver DNA of rats given near toxic doses of carbon tetrachloride, ethanol, yellow phosphorus, bromobenzene, thioacetamide, puromycin, and N-nitrosopyrrolidine, but not diethylnitrosamine. The DNA methylation response appears to be capricious except for hydrazine and N-nitrosopyrrolidine.

PREFACE

This is the annual report of the subprogram on Comparative Biochemistry and Metabolism, Part 1: Carcinogenesis and concerns work performed by the Department of Community and Environmental Medicine of the University of California, Irvine, on behalf of the Air Force under Contract Number F33615-80-C-0512, Work Unit 63020115. This document describes the accomplishments of the subprogram from June 1981 through May 1982.

R. C. Shank, Ph.D., was principal investigator for the subprogram. Acknowledgement is made to E. J. Hunt and W. F. Pool for their significant research contributions and assistance in the preparation of this report. M. Pinkerton, Toxicology Branch, was the technical monitor for the Aerospace Medical Research Laboratory.

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INTRODUCTION

Hydrazine (H_2N-NH_2) is a strong reducing agent which has widespread use as an anticorrosive, an intermediate in chemical syntheses, and as a propellant fuel. At high doses it is neurotoxic to mammals, rapidly producing convulsions and coma; at lower doses the compound is toxic to the liver, producing fatty accumulation and focal and periportal necrosis. High levels of hydrazine sulfate in the drinking water of rats have produced hepatocellular carcinomas (Severi and Biancifiori, 1968); inhalation of the free base at levels irritating to the respiratory tract produced squamous cell carcinomas in the nasal turbinates of rats (MacEwen et al., 1979). Biancifiori and Ribacchi (1962) reported an increase in the incidence of pulmonary carcinomas in mice given hydrazine sulfate in the drinking water. Hydrazine appears to be a carcinogen only at doses which produce cytotoxicity.

The present studies were an attempt to provide biochemical information which can be useful in determining the mechanism by which hydrazine, and perhaps other agents which appear to be carcinogenic only after repeated cytotoxic administrations, induce cancer. Previous studies in this laboratory have demonstrated that administration of near LD_{50} amounts of hydrazine to rats and mice results in the rapid formation of 7-methylguanine and O^6-methylguanine in liver DNA (Barrows and Shank, 1978; Barrows and Shank, 1980; Shank et al., 1980; Shank, 1981; Becker et al., 1981). These reports have also given evidence that this response may be true for other hepatotoxins as well and therefore may not be specific to hydrazine itself. The source of the methyl moiety in the methylation of liver DNA in this process appears to be endogenous S-adenosylmethionine. The pattern of methylation in DNA is qualitatively similar to that seen after the administration of strong alkylating carcinogens such as dimethylnitrosamine, 1,2-dimethylhydrazine, or methylnitrosourea.

The present report summarizes further work done on the kinetics of the methylation of DNA guanine in liver after administration of hydrazine to the rat and hamster, the possible role monomethylhydrazine may play in the hydrazine-stimulated methylation of DNA, and the importance of the hepatic pool of glutathione in the S-adenosylmethionine-dependent methylation process.

RESEARCH PROGRAM

METHODS DEVELOPMENT

Fractionation of DNA Hydrolysates by Liquid Chromatography

Highly polymerized DNA was isolated and purified by the phenol extraction method of Kirby (1962) as modified by Swann and Magee (1968). In some cases the DNA was hydrolyzed directly in 0.1 M HCl (5 mg DNA/ml) for 30-40 minutes at 70°C to yield pyrimidine oligonucleotides and purines as free bases. In the majority of cases, however, the DNA was first dissolved in 10 mM sodium cacodylate pH 7.00 (5 mg DNA/ml) and heated to 100°C for 35 minutes to release 7-methylguanine from the DNA polymer; the partially apurinic DNA was precipitated from the neutral hydrolysate by adding 0.11 volume 1 M HCl and hydrolyzed as above.

Neutral thermal hydrolysates containing 7-methylguanine and small amounts of guanine and adenine were fractionated on a 25 cm preparative Partisil 10 Magnum 9 strong cation exchange column (Whatman, Clinton, NJ) with a two-step gradient of water for 4 minutes and then 0.1 M $NH_4H_2PO_4$ pH 2.0 at a flowrate of 4 ml/minute. Hydrolysates in 0.1 M HCl were fractionated on a 25-cm analytical Partisil 10 strong cation exchange column isocratically with 0.1 M $NH_4H_2PO_4$ pH 2.0 at 2 ml/minute. In both cases elution of bases was monitored by fluorescence spectrophotometry at an excitation wavelength of 286 nm and using an interference emission filter at 344 nm. Quantitation was achieved using a Hewlett Packard 3380S electronic reporting integrator which had been calibrated by injecting known quantities of authentic guanine, 7-methylguanine, and O^6-methylguanine onto the column. These methods have been described in detail previously (Herron and Shank, 1979; Barrows and Shank, 1980; Becker et al., 1981).

Glutathione Assay

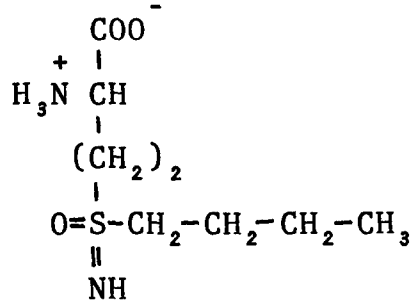
Hepatic levels of reduced glutathione were measured according to the method of Ellman (1959) which determines the total non-protein reduced sulfhydryl content; in the case of liver homogenate, this is 99% glutathione. Liver was homogenized in cold water (4 g/ml) and three 1 ml aliquots were mixed with cold 4% sulfosalicylic acid (1 ml to 1 ml) to precipitate protein; the remainder of the homogenate was used for DNA isolation. After centrifugation 0.5 ml of the supernatant was mixed with 4.5 ml freshly prepared 5,5'-dithio-bis-2-nitrobenzoic acid to develop the chromophore. Absorbance at 412 nm was determined and compared to that of standard solutions of glutathione handled in an identical manner. Results were expressed as µg glutathione/g liver.

S-Adenosylmethionine Assay

S-Adenosylmethionine in liver was determined quantitatively by the high pressure chromatographic method of Barrows and coworkers (1982). Liver was homogenized in an equal volume of cold water, and protein and nucleic acids were precipitated with a volume of 15% trichloroacetic acid equal to two volumes of liver. Six ml of the soluble fraction was mixed with 1.5 ml 40% ammonium reineckate in dimethylformamide. The reineckate complexes were collected by centrifugation and extracted three times with water and ether:methylethylketone (1:2). The aqueous phase was analysed directly by application to a 25 cm analytical Partisil 10 strong cation exchange column (Whatman, Clinton, NJ) and eluting S-adenosyl-homocysteine, methylthioadenosine, and S-adenosylmethionine (in that order) isocratically with 0.1 M $NH_4H_2PO_4$ pH 3.00 at 2 ml/minute. Elution was monitored at 254 nm and the amount of S-adenosyl-methionine was quantified by peak height analysis and comparison to calibration curves derived with standard S-adenosylmethionine (repurified from Sigma, St. Louis, MO).

Preparation of Buthionine Sulfoximine

Buthionine sulfoximine, a specific inhibitor of glutathione synthetase I, was synthesized according to the method of Griffith and Meister (1979). DL-Homocysteine thiolactone (68 g, 0.43 mol, Sigma Chemical Co., St. Louis, MO) in 1 liter of freshly prepared sodium methoxide was stirred under N_2 for 30 minutes; 87 g 1-iodobutane (0.47 mol, Aldrich Chemical Co., Milwaukee, WI.) were added, and the reaction mixture was stirred overnight. The filtered mixture was reduced in volume to ca. 250 ml under vacuum, 500 ml 4 M sodium hydroxide was added to the concentrated solution, and the mixture was refluxed for 6 hours. The filtered mixture was neutralized with concentrated HCl to precipitate DL-buthionine, and 30 mmol of this product was stirred at 55°C with a mixture of 60 ml $CHCl_3$ and 17 ml concentrated H_2SO_4. Sodium azide (140 mmol; Alfa Products, Danvers, MA) was added in small portions over the 12-hour mixing process. After cooling, the mixture was washed with cold water; the water washings were applied to a Dowex-50 8X H^+ strong cation exchange column (2.5 x 40 cm, 200 mesh), the column was washed with water, and the buthionine sulfoximine was eluted with 3 M ammonium hydroxide. The eluate was concentrated and the buthionine sulfoximine was crystallized by the addition of cold ethanol. The yield was 67%. After 5 recrystallizations the buthionine sulfoximine had a melting point of 213-214°C (published m.p. 214-215.5°C).



buthionine sulfoximine
(S-n-butyl homocysteine sulfoximine

Preparation of 7-Methyldeoxyguanosine

7-Methyl-2'-deoxyguanosine was prepared by the method of Jones and Robins (1963). 2'-Deoxyguanosine (250 mg, Calbiochem, La Jolla, CA) was dissolved in 2.5 ml dimethylsulfoxide at 50°C. The solution was cooled to 20°C and 0.3 ml methyl iodide added. The mixture was held at 20°C for 1 hour with stirring and then gradually warmed to 28°C over the next 3.5 hours. Excess methyl iodide was removed by evaporation at room temperature. The reaction mixture was cooled to 15°C and 19.8 ml chloroform at 15°C were added. The solution was held at 10-15°C for 3 hours, and the precipitate that formed was collected by filtration, washed twice with ethanol and twice with ether. The yield was 124 mg.

Five milligrams of crude product were dissolved in 5 ml water and analyzed by high pressure liquid chromatography. The solution was fractionated on a Whatman Partisil 10, 25 cm analytical strong cation exchange column using 10 mM ammonium phosphate pH 4.0 at 3 ml/minute; elution was monitored at 275 nm. One major and two minor components were detected with retention volumes of 40.5, 28.5, and 21 ml, respectively. Standard 7-methylguanine and 7-methylguanosine (Sigma Chemical, Co., St. Louis, MO) had retention volumes of 21 and 28.5 ml, respectively. The major component was purified by high pressure liquid chromatography, and ultraviolet spectra at pH 1 and pH 7 were determined on a Cary 210 spectrophotometer. The spectra were similar to those reported by Jones and Robins (1963). Heating the purified product at 100°C pH 1 for 10 minutes destroyed the putative 7-methyldeoxyguanosine and yielded material which cochromatographed with authentic 7-methylguanine. Equimolar amounts of putative 7-methyldeoxyguanosine and deoxyguanosine (Sigma Chemical Co.) were reacted with diphenylamine to form the characteristic chromophore; both preparations had the same absorbance at 595 nm. Thus, presumptive evidence supports the successful synthesis of 7-methyl-2'-deoxyguanosine and its separation from 7-methylguanosine by liquid chromatography.

Fractionation of Nucleosides from Enzymatic Hydrolysates of Liver DNA

DNA was dissolved in 10 mM magnesium acetate pH 6.5 (5 mg/ml) and depolymerized with DNase I (100 units/ml; Sigma Chemical Co.) at 37°C for 30 minutes. The solution was adjusted to pH 8.5 with 1 M Tris buffer, and the DNA was digested with venom phosphodiesterase (0.03 units/ml) and alkaline phosphatase (3 units/ml) at 37°C for 16 hours. Hydrolysis was not complete but sufficient to release large quantities of thymidine (TdR), deoxycytidine (CdR), deoxyguanosine (GdR), deoxyadenosine (AdR), and 5-methyldeoxycytidine (5-Me-CdR), which were separated on a 25-cm preparative strong cation exchange column with water (first 20 ml) and then 15 mM ammonium phosphate pH 3.5 at 5 ml/minute. Elution volumes were: TdR = 58 ml, GdR = 70.7 ml, CdR = 114.3 ml, AdR = 122.6 ml, 5-Me-CdR = 181.4; added standard 7-methylguanosine (7-Me-Gu) eluted immediately after 5-Me-CdR with an elution volume of 197.1 ml, and 7-methyldeoxyguanosine followed as a fully resolved but broad elution peak. It is necessary to identify the elution characteristics of 7-Me-Gu in order to detect RNA contamination in DNA.

Purity of Toxicants

The purity of the hydrazine and monomethylhydrazine used in these studies was evaluated by gas chromatography - mass spectrometry (gc-ms). The purity of hydrazine was greater than 99%; slightly less than 1% of the hydrazine sample had a mass spectrum consistent with that of m-cresol, a compound used in the commercial synthesis of hydrazine. The commercial hydrazine was analyzed by gc-ms specifically for monomethylhydrazine, 1,2-dimethylhydrazine, and dimethylnitrosamine and was found to be free of these methylating intermediates. Monomethylhydrazine was found to be greater than 99% pure by gc-ms and no impurities could be detected. Hydrazine was obtained from Matheson (Norwood, OH) and monomethylhydrazine was purchased from Aldrich Chemical Co. (Milwaukee, WI).

METHYLATION OF LIVER DNA AFTER HYDRAZINE ADMINISTRATION

Dose- and Time-Response Relationships in the Rat and Hamster

One possible explanation for why DNA may appear aberrantly methylated in response to hydrazine toxicity may be due to impairment of the normal DNA "repair" process by hydrazine (Becker et al., 1981). It has recently been shown that mammalian liver contains proteins which are able to remove 7-methylguanine and O^6-methylguanine from DNA (Laval et al., 1981; Singer and Brent, 1981; Margison and Pegg, 1981); the hamster has a greater activity of liver 7-methylguanine glycosylase and a low activity for removal of

O^6-methylguanine from DNA (Stumpf et al., 1979; Margison and Pegg, 1981) compared to the rat.

A series of experiments was carried out to compare the hamster with the rat in their abilities to methylate and demethylate liver DNA guanine in response to hydrazine administration in preparation for studies on the effect of hydrazine on 7-methylguanine glycosylase and O^6-methylguanine repair proteins.

Hydrazine was administered by stomach tube in 0.1 M HCl (0.1 ml per 100 g body weight) to Syrian golden hamsters (70-100 g) or Fischer 344 rats (150-200 g) obtained from Charles River Breeding Laboratory, MA. Levels of 7-methylguanine and O^6-methylguanine were determined by fluorescence HPLC at various times following administration of hydrazine at one of four doses and compared to those determined earlier in the rat (Shank, 1981). The data are summarized in Tables 1-4; results of the rat experiments have been published (Becker et al., 1981).

TABLE 1. METHYLATION OF LIVER DNA IN HAMSTERS TREATED WITH HYDRAZINE

Dose (mg/kg BW)	DNA Methylation (μmol methylguanine/mol guanine) 7-Methylguanine		O^6-methylguanine		Ratio O^6MG:7MG
0	ND[a]		ND[b]		
0	ND		ND		
45	180		36		
45	200		22		
		mean = 190		mean = 29	0.15
60	256		67		
60	258		66		
		mean = 257		mean = 67	0.26
75	267		46		
75	334		81		
		mean = 301		mean = 64	0.21
90	203		33		
90	347		87		
		mean = 275		mean = 60	0.22

[a] no 7-methylguanine detected (less than 50 μmol 7-methylguanine/mol guanine

[b] no O^6-methylguanine detected (less than 2 μmol O^6-methylguanine/mol guanine

TABLE 2. VARIATION WITH TIME IN LEVELS OF 7-METHYLGUANINE AND O^6-METHYLGUANINE IN LIVER DNA AFTER SINGLE ADMINISTRATION OF 90 MG HYDRAZINE/KG BODY WEIGHT TO HAMSTERS

Time after admin. (hr)	DNA Methylation (μmol methylguanine/mol guanine) 7-methylguanine	O^6-methylguanine	O^6MG:7MG
0	ND[a]	ND[b]	
	ND	ND	
0.25	162	21	
	135	14	
	mean = 149	mean = 17	0.11
0.50	477	32	
	344	27	
	mean = 410	mean = 30	0.07
1	364	23	
	383	32	
	mean = 373	mean = 27	0.07
6	649	48	
	905	88	
	mean = 777	mean = 68	0.09
12	643	83	
	228	42	
	mean = 435	mean = 63	0.14
24	424	63	
	507	88	
	mean = 466	mean = 76	0.16
48	310	58	
	406	95	
	mean = 358	mean = 77	0.22
72	141	27	
	251	30	
	mean = 196	mean = 29	0.15

[a] no 7-methylguanine detected (less than 50 μmol 7-methylguanine/mol guanine)

[b] no O^6-methylguanine detected (less than 2 μmol O^6-methylguanine/mol guanine).

TABLE 3. METHYLATION OF LIVER DNA GUANINE 24 HOURS AFTER ADMINISTRATION OF VARIOUS DOSES OF HYDRAZINE TO RATS

Dose[a], mg/kg	DNA Methylation (μmol methylguanine/mol guanine) 7-methylguanine		O^6-methylguanine		O^6MG:7MG
0	ND[b]		ND[c]		
	ND		ND		
45	419		14		
	422		16		
		mean = 421		mean = 15	0.04
60 (ca. $LD_{0.01}$)	398		12		
	724		29		
		mean = 561		mean = 20	0.04
75	564		19		
	498		25		
		mean = 531		mean = 22	0.04
90 (ca. LD_{50})	794		48		
	944		68		
		mean = 869		mean = 58	0.07

[a] lethal doses estimated from dose-lethality data by the method of Litchfield and Wilcoxon (1949)

[b] no 7-methylguanine detected (less than 50 μmol 7-methylguanine/mol guanine)

[c] no O^6-methylguanine detected (less than 2 μmol O^6-methylguanine/mol guanine)

TABLE 4. VARIATION WITH TIME IN THE LEVELS OF 7-METHYLGUANINE AND O^6-METHYLGUANINE IN LIVER DNA AFTER THE SINGLE ORAL ADMINISTRATION OF 90 MG HYDRAZINE/KG BODY WEIGHT TO RATS

Time After Admin. (hr)	DNA Methylation (μmol methylguanine/mol guanine) 7-methylguanine	O^6-methylguanine	O^6MG:7MG
0	ND[a]	ND[b]	
	ND	ND	
0.25	213	11	
	409	19	
	mean = 311	mean = 15	0.05
0.50	381	17	
	912	33	
	mean = 647	mean = 25	0.04
1	434	35	
	758	53	
	mean = 596	mean = 44	0.07
6	500	42	
	1,240	112	
	mean = 870	mean = 77	0.09
12	902	91	
	767	68	
	mean = 835	mean = 80	0.10
24	838	52	
	885	82	
	mean = 862	mean = 67	0.08
48	600	14	
	637	21	
	mean = 619	mean = 18	0.03
72	355	trace	
	558	trace	
	mean = 447		-
96	234	trace	
	273	ND	
	mean = 259		-

[a] no 7-methylguanine detected (less than 50 μmol 7-methylguanine/mol guanine)

[b] no O^6-methylguanine detected (less than 2 μmol O^6-methylguanine/mol guanine)

The amounts of 7-methylguanine in hamster liver DNA were less than in rat liver DNA for the same doses of hydrazine, but the opposite is true for O^6-methylguanine levels; the greater levels of O^6-methylguanine in the hamster probably reflect the slower rate of removal of this base from DNA in this species (Stumpf et al., 1979). The net effect is that the ratio of O^6-methylguanine to 7-methylguanine in hamster liver (0.15-0.26) is considerably greater than in the rat (0.01-0.07) following hydrazine administration.

The rates of formation of the methylguanines in liver DNA in response to hydrazine administration were the same for both the rat and hamster; however, the rate at which O^6-methylguanine was removed from liver DNA in the hamster was considerably slower than that in the rat (Figure 1); this is in agreement with the results of Stumpf and coworkers (1979) on the formation and removal of methylguanines in liver DNA of rats and hamsters given a single administration of dimethylnitrosamine. The kinetics of DNA methylation in hydrazine toxicity, then, appear to be the same as those for methylating carcinogens. This is further evidence supporting the identity of 7-methylguanine and O^6-methylguanine as the methylated bases in these studies with hydrazine.

Comparative Methylation of Liver DNA in Mice and Hamsters Given Hydrazine or Monomethylhydrazine

Perhaps the most obvious mechanism which can be proposed to explain DNA methylation in animals given hydrazine is as follows: hydrazine in the liver cell may be the substrate for methylation by an N-methyltransferase which uses S-adenosylmethionine for the source of the methyl moiety; the product, monomethylhydrazine, could then be metabolically activated to a methylating agent, as shown by Hawks and Magee (1974). If monomethylhydrazine is the intermediate in DNA methylation in hydrazine-treated animals, then administration of monomethylhydrazine, the more proximal alkylating intermediate, might be expected to be more efficient in the methylation of DNA, especially when Dost and coworkers (1979) have shown that 75% of the hydrazine administered to rats can be accounted for as expired N_2 and urinary acetylated hydrazine and thus is not available for methylation to monomethylhydrazine.

Mice given equimolar (0.33 mmol) doses of hydrazine (10.4 mg/kg body weight) or monomethylhydrazine (15 mg/kg body weight) and hamsters given 45, 60, 75 or 90 mg hydrazine/kg body weight or 11.3, 14.1, 17.6, 22, or 27.5 mg monomethylhydrazine/kg body weight were used to compare DNA methylation levels produced by the two compounds. The results, summarized in Table 5, show clearly that more methylation of liver DNA results from the administration of the hepatotoxin, hydrazine, than from monomethylhydrazine (which is not

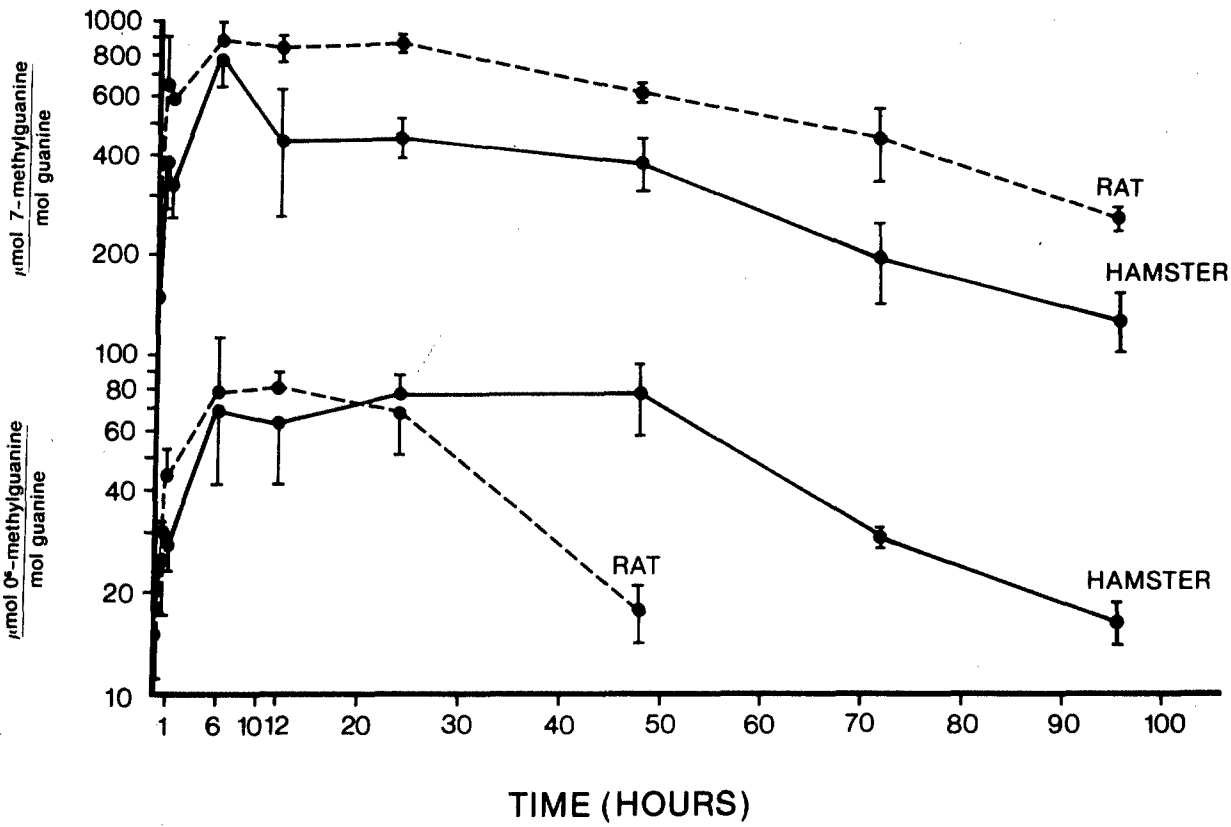


Figure 1. A semilogarithmic plot of methylguanine concentrations vs. time in order to demonstrate the rate of formation and persistence in rat and hamster liver DNA.

TABLE 5. METHYLATED PURINES IN LIVER DNA AFTER TREATMENT OF MICE AND HAMSTERS WITH HYDRAZINE OR MONOMETHYLHYDRAZINE

Species	Agent (mg/kg)	DNA Methylation (μmol methylguanine/mol guanine) 7-methylguanine	O^6-methylguanine
Mouse[a]	Hydrazine (10.4)	157	9
	MMH (15)	52	2
Hamster[b]	Hydrazine (45)	190	29
	(60)	257	67
	(75)	301	64
	(90)	275	60
	MMH, all doses	ND[c]	ND

[a] mice killed 1 hour after toxicant administration.

[b] hamsters killed 24 hours after toxicant administration.

[c] no methylguanine detected (less than 50 μmol 7-methylguanine or 2 μmol O^6-methylguanine/mol guanine)

hepatotoxic), suggesting that monomethylhydrazine is not an important intermediate in DNA methylation in animals poisoned with hydrazine.

Effect of Glutathione Depletion on Liver DNA Methylation in Hydrazine-Treated Hamsters

A study was initiated to determine whether depletion of hepatic glutathione alters the methylation of liver DNA in response to administration of a toxic dose of hydrazine. If hepatotoxicity results in an intracellular redistribution of S-adenosylmethionine such that this biochemical methylating agent may randomly and non-enzymatically methylate nucleophiles in the cell (hence DNA methylation), then perhaps glutathione serves as a scavenger in normal hepatocytes to protect sensitive nucleophiles such as nucleic acids. Depletion of hepatic glutathione prior to administration of hydrazine may, then, increase the extent of methylation of liver DNA.

Thirty young male Syrian golden hamsters (Simonsen Labs., Gilroy, CA) were divided into 5 groups:

a) 0.1 M HCl, 0.1 ml orally per 60 g body weight.
b) 90 mg hydrazine in 0.1 M HCl, 0.1 ml orally per 60 g body weight.

c) corn oil, 0.1 ml intraperitoneally per hamster
d) 60 µl diethylmaleate in 0.1 ml oil, intraperitoneally, 0.1 ml per hamster
e) 60 µl diethylmaleate intraperitoneally, followed 30 min later by 90 mg hydrazine/kg body weight

Time = 0; diethylmaleate or corn oil injected
Time = 0.5 hour = hydrazine or HCl injected
Time = 1.5 hours = kill by decapitation

Immediately after decapitation, the livers were excised and washed in ice cold saline and frozen at -70°C. Assays for reduced glutathione were performed on each liver and the quantitative determination of the amounts of 7-methylguanine and O^6-methylguanine were made on DNA isolated from pools of 3 livers each.

Diethylmaleate pretreatment decreased the level of hepatic glutathione by 83%; hydrazine alone decreased glutathione levels in the liver by 11%. The results are summarized in Table 6.

TABLE 6. NON-PROTEIN SULFHYDRYL (GLUTATHIONE) LEVELS IN LIVERS OF HAMSTERS TREATED WITH DIETHYLMALEATE (DEM) AND/OR HYDRAZINE

Treatment	mg GSH/g liver ± S.D.	
HCl control	2.73 ± 0.18	
Oil control	2.86 ± 0.09	
Hydrazine only	2.44 ± 0.21	(89% of control)
DEM only	0.50 ± 0.33	(17% of control)
DEM, then hydrazine	0.30 ± 0.05	(11% of control)

The only DNA samples found to contain 7-methylguanine and O^6-methylguanine (in readily detectable amounts) were those prepared from animals treated with hydrazine only. Rather than finding the expected increased level of DNA methylation in diethylmaleate-pretreated animals, no aberrant methylation was detected. Since diethylmaleate scavenges free sulfhydryl groups, it may be that diethylmaleate scavenges not only the reduced glutathione but S-adenosylhomocysteine as well; depletion of the S-adenosyl-homocysteine pool would in turn deplete the rapidly turning-over S-adenosylmethionine, the putative methylating agent in hydrazine toxicity. In a subsequent experiment diethylmaleate treatment had little effect on the level of S-adenosylmethionine in the liver. It is also possible that diethylmaleate reacted directly with hydrazine, protecting the liver from the toxic action of that reducing agent.

A more specific inhibitor of glutathione formation was sought and found. Buthionine sulfoxime (BS) has been reported to be a highly specific inhibitor of glutathione synthetase I, the enzyme that forms the dipeptide in the first step of the synthesis of glutathione (Griffith and Meister, 1979). This material was prepared in the laboratory and given intraperitoneally to two hamsters; in one animal the glutathione level in liver was depressed 80% compared to untreated animals, but in the second animal no change in glutathione level was detected.

Another trial was made in which 4 mmol BS/kg body weight was given orally to 8 rats as a solution in 0.1 M HCl, rather than a suspension in water intraperitoneally as was done above; another 8 animals were given HCl only. Six hours after BS administration 4 animals in each group were given 0.1 M HCl or 90 mg hydrazine per kilogram body weight orally; the remaining 4 animals in each group were given HCl only. All animals were decapitated 1 hour later. Individual livers were analyzed for 1) the concentration of reduced glutathione, 2) the concentration of S-adenosylmethionine, and 3) the concentration of 7-methylguanine and O^6-methylguanine in DNA. The results are summarized in Table 7. Although BS was effective in depleting hepatic glutathione levels (GSH levels in treated animals were approx. 45% those in control), there was no effect of BS on the levels of S-adenosylmethionine or on the levels of 7-methylguanine and O^6-methylguanine in liver DNA. It would seem that hepatic glutathione levels do not play a critical role in the S-adenosyl-methionine-mediated methylation of liver DNA in hydrazine-poisoned rats.

TABLE 7. EFFECT OF BUTHIONINE SULFOXIMINE (BS) ON HEPATIC LEVELS OF GLUTATHIONE (GSH) AND S-ADENOSYLMETHIONINE (SAM) AND ON LIVER DNA METHYLATION IN HYDRAZINE-POISONED RATS

Agents	SAM (nmol/g liver)	GSH (μg/g liver)	7-methylguanine (μmol/mol G)	O^6-methylguanine (μmol/mol G)
HCl/HCl	7.63 ± 0.86	1247 ± 89	ND[a]	ND[b]
BS/HCl	6.35 ± 0.67	565 ± 50	ND	ND
HCl/HZ	6.85 ± 1.63	1196 ± 234	462 ± 184	29 ± 11
BS/HZ	7.82 ± 1.57	449 ± 48	432 ± 137	30 ± 9

HCl, 0.1 M HCl; BSO, 4 mmol buthionine sulfoximine/kg body wt.; HZ, 90 mg hydrazine/kg body wt.

[a] no 7-methylguanine detected (less than 50 μmol 7-methylguanine/mol guanine

[b] no O^6-methylguanine detected (less than 2 μmol O^6-methylguanine/mol guanine

Effect of Ethionine on DNA Methylation in Hydrazine-poisoned Rats

A preliminary experiment (Shank et al., 1980) was carried out to determine whether the methionine analog, ethionine, which is a known inhibitor of S-adenosylmethionine synthesis, was able to block the methylation of liver DNA in response to hydrazine administration; it is thought that this methylation process is mediated by endogenous S-adenosylmethionine. In the preliminary experiment, animals were pretreated with ethionine, and liver DNA methylation was measured as the incorporation of tritium from (^{3}H-methyl)methionine into 7-methylguanine fractions of hydrolyzed DNA. It was found that immediate ethionine pretreatment prevented the incorporation of tritium into 7-methylguanine in hydrazine-poisoned rats, but that delayed (12 hours) pretreatment was without effect.

That preliminary experiment was repeated, using the optical technique for quantitating the amounts of methylguanines in DNA. Male Fischer 344 (140-180 g, Charles River) rats were fasted overnight. In one experiment, the animals were given saline or 500 mg ethionine/kg body weight in saline intraperitoneally at 3:00 p.m.; 12 hours later the rats were given 0.1 M HCl or 60 mg hydrazine/kg body weight in 0.1 M HCl per os and killed 5 hours later. In the second experiment, the animals were given the ethionine/saline at 10 a.m. and the hydrazine/HCl at 10:15 a.m. and killed 3 hours later (1:15 p.m.). Liver DNA was isolated from pairs of livers and assayed for 7-methylguanine and O^6-methylguanine by the standard HPLC-fluorescence technique. The results of these experiments are summarized in Table 8. The results confirm those obtained in the preliminary experiment using radioactivity, namely, immediate, but not delayed, pretreatment with ethionine inhibits to some extent the methylation of liver DNA in rats given hydrazine. This effect appears to be more pronounced in the methylation of guanine at the O^6-position, and this seems to be the first instance in which we have found a separation in the methylation of guanine at the 7- and O^6-positions, if indeed this effect is real. At this dose of ethionine, the hepatic pool of S-adenosylmethionine is rapidly depleted (within 15-30 minutes); at 12 hours the S-adenosylmethionine levels are still low, and the S-adenosylethionine levels are large and inhibit a variety of S-adenosylmethionine-dependent enzymes. The results of this experiment then suggest that ethionine does seem to inhibit the methylation of liver DNA in hydrazine-treated rats, but probably not via inhibition of an S-adenosylmethionine-dependent enzyme, but perhaps by depleting the hepatic level of S-adenosylmethionine itself, arguing in favor of a direct methylation of DNA by this endogenous intermediate.

TABLE 8. EFFECT OF ETHIONINE ON THE METHYLATION OF LIVER DNA IN RATS GIVEN HYDRAZINE

Ethionine Pretreatment	Treatment	DNA Methylation (μmol methylguanine/mol G) 7-methylguanine	O^6-methylguanine
12 hours before hydrazine administration	Saline-Hydrazine	302	31
		318	30
		$\bar{x}$ = 310	$\bar{x}$ = 31
	Ethionine-Hydrazine	287	20
		306	22
		$\bar{x}$ = 297	$\bar{x}$ = 21
0.25 hour before hydrazine administration	Saline-Hydrazine	355	39
		289	21
		$\bar{x}$ = 322	$\bar{x}$ = 30
	Ethionine-Hydrazine	276	21
		151	16
		$\bar{x}$ = 213	$\bar{x}$ = 18

VARIOUS HEPATOTOXINS AND METHYLATION OF LIVER DNA

Methylation of Liver DNA Following Administration of Various Hepatotoxins to Rats

A series of experiments have been carried out on DNA methylation in rat liver during hepatotoxicity brought about by administration of single, high doses of a variety of hepatotoxins, in addition to hydrazine, to determine whether this methylation response is specific to hydrazine or representative of a non-specific response to cytotoxicity. The toxic agents used in this series included carbon tetrachloride (CCl_4), ethanol (CH_3CH_2OH), yellow phosphorus (P), bromobenzene (C_6H_5Br), thioacetamide (CH_3CSNH_2), puromycin (an antibiotic) and N-nitrosopyrrolidine (C_4H_8N-NO). The net results of this experimental series are summarized in Table 9; each toxicant was administered orally or intraperitoneally (single dose) at 1/3 - 1 published LD_{50}, and the animals were killed at near the time of maximum DNA methylation levels. It is clear from Table 9 that the DNA methylation response appears capricious except for hydrazine and N-nitrosopyrrolidine. Even the gross appearance of the liver in these experiments has been difficult to reproduce readily. These experiments have involved several animal groups per trial with small numbers of animals per group. Administration of 1/2 the LD_{50} of

TABLE 9. LIVER DNA METHYLATION IN RESPONSE TO ADMINISTRATION OF VARIOUS HEPATOTOXINS TO THE RAT

Chemical	Methylguanines in Liver DNA (μmol/mol guanine) 7-Methylguanine	O^6-Methylguanine
Hydrazine	865 ± 64	63 ± 16
CCl_4	560; trace; ND[a]	14; ND; ND[b]
Ethanol	389; trace; ND	ND; ND; ND
Bromobenzene	ND; 59	ND; ND
Phosphorus	ND; 72	ND; ND
Puromycin	ND; 110	ND; ND
N-Nitrosopyrrolidine	104; 120	ND; ND

± S.D. N = 4; each value represents the result of an independent experiment.

[a] no 7-methylguanine detected (less than 50 μmol 7-methylguanine/mol guanine

[b] no O^6-methylguanine detected (less than 2 μmol O^6-methylguanine/mol guanine

N-nitrosopyrrolidine results in the reproducible formation of 7-methylguanine in liver DNA in rats; when the liver carcinogen is labeled as (^{14}C-2,5)N-nitrosopyrrolidine, the 7-methylguanine resultant in the DNA is not radioactive; on the other hand if unlabeled N-nitrosopyrrolidine and (^{3}H-methyl)methionine are administered simultaneously, the 7-methylguanine which forms in DNA is tritium-labeled (Hunt and Shank, 1982). Diethylnitrosamine, which is toxic to the liver, did not stimulate methylation of DNA in any one of several studies on this potent carcinogen. Nevertheless, it remains that one is able to stimulate formation of 7-methylguanine in liver DNA by administration of high doses of several non-alkylating hepatotoxins.

IDENTIFICATION OF O^6-METHYLGUANINE

Confirmation of Identity of O^6-Methylguanine in DNA from Rats Given Hydrazine

The experimental evidence obtained to identify an aberrant base in liver DNA as O^6-methylguanine has included a) cochromatography of the suspect material with authentic O^6-methylguanine in five liquid chromatography systems, b) thermal stability characteristics of the base in DNA in acid and neutral solution, c) fluorescence spectral properties, d) dependence on methionine for the methyl source, and e) its rate of removal from the DNA polymer *in vivo*. Another study

was completed which offers even greater evidence that the material identified as O^6-methylguanine is indeed that compound.

Dr. Peter F. Swann of the Courtauld Institute of Biochemistry, Middlesex Hospital, University of London, developed a sensitive and specific radioimmunoassay for O^6-methyldeoxyguanosine and kindly offered to analyze our DNA samples to confirm the identity of this methylated base.

Sprague Dawley rats were given orally 90 mg hydrazine/kg body weight in 0.1 M HCl and killed 24 hours later by decapitation. Liver DNA was isolated and a portion was sent to Dr. Swann for radioimmunoassay; the remainder was kept in the laboratory at UCI, and later analyzed for the presence of 7-methylguanine and O^6-methylguanine by the usual fluorescence liquid chromatography assay and shown to contain only approximately 13% of the O^6-methylguanine expected for a dose of 90 mg hydrazine/kg body weight. Dr. Swann confirmed the identity of O^6-methylguanine but quantitatively detected only 17% of the amount of O^6-methylguanine he was told should have been in the particular sample (before the DNA was analyzed in our laboratory); thus the results of Dr. Swann confirm qualitatively (and quantitatively) that hydrazine administration to rats results in the formation of O^6-methylguanine in liver DNA. Later, Dr. Swann treated rats with hydrazine in his own laboratory, isolated the liver DNA, and demonstrated again the presence of O^6-methylguanine using the radioimmunoassay.

SUMMARY AND CONCLUSIONS

Comparative carcinogenesis and DNA alkylation studies have demonstrated the importance in cancer induction of both cellular proliferation and persistence of O^6-alkylguanine molecules in target-organ DNA (Goth and Rajewsky, 1974; Craddock, 1976). The formation and persistence of 7-methylguanine, and particularly O^6-methylguanine, in liver DNA of rats treated with hydrazine, in conjunction with restorative hepatic hyperplasia induced by this toxicant, may be related analogously to hydrazine hepatocarcinogenicity. Carbon tetrachloride and ethanol, chemicals which are also thought to be carcinogenic only when administered at near-toxic doses over long periods (Reuber and Glober, 1970; Lieber et al., 1979), also appear capable of producing 7-methylguanine, and perhaps O^6-methylguanine, in liver DNA, presumably via mechanisms similar to those applicable to hydrazine (Shank and Barrows, 1981; Barrows and Shank, 1980; Barrows and Shank, 1981).

It is possible that hydrazine may be metabolically methylated by S-adenosylmethionine to form monomethylhydrazine; monomethylhydrazine is known to be enzymatically oxidized to a compound capable of

methylating liver DNA *in vivo* (Hawks and Magee, 1974). Several lines of evidence, however, suggest that monomethylhydrazine is not an important intermediate in hydrazine-induced DNA methylation. Rat liver DNA was found to be methylated to a greater extent in hydrazine-treated rats than in monomethylhydrazine-treated animals, following treatment with equitoxic doses of these chemicals (Shank et al., 1980); similar results from current studies in mice and hamsters given equimolar doses of the two compounds also failed to support monomethylhydrazine as an important intermediate in hydrazine-stimulated DNA methylation. Moreover, 15 minutes after hydrazine administration to rats and hamsters liver DNA contained appreciable quantities of 7-methylguanine and O^6-methylguanine; in fact, the time to half-maximum methylation levels was only 30-45 minutes. This methylation process would not be expected to occur so rapidly if hydrazine is first enzymatically methylated to monomethylhydrazine, and then the monomethylhydrazine is oxidatively metabolized to the ultimate methylating species. These results, therefore, continue to be inconsistent with the suggestion that monomethylhydrazine is the more proximal methylating agent involved in hydrazine hepatotoxicity-induced liver DNA methylation.

S-Adenosylmethionine continues to remain the major candidate for the biochemical intermediate in the DNA methylation response. If S-adenosylmethionine synthesis in the liver is inhibited by the methionine analog, ethionine, hydrazine administration fails to stimulate DNA methylation; as S-adenosylmethionine levels return to normal several hours after ethionine administration, the hydrazine - DNA methylation response returns as well, in spite of the fact that the hepatocytes contain large amounts of S-adenosylethionine which inhibit S-adenosylmethionine - dependent methyltransferases, such as the enzyme that normally methylates DNA in the 5-position of cytosine. This suggests, then, that hydrazine may stimulate a non-enzymatic methylation of liver DNA by S-adenosylmethionine.

Barrows and Magee (1982) have shown that naked DNA incubated with purified S-adenosylmethionine is rapidly methylated at the 7-position of guanine without the need of any cofactors or enzymes; their methods were not sufficiently sensitive to detect O^6-methylguanine at levels of one-tenth those at which 7-methylguanine occurred.

In the present studies it was proposed that S-adenosylmethionine in the normal hepatocyte had to be sequestered or scavenged to prevent unwanted methylations from occurring in the cell. A likely endogenous scavenger of free S-adenosylmethionine in the hepatocyte would be reduced glutathione. It was also proposed that a mechanism by which hydrazine might bring about DNA methylation could be the depletion of hepatic stores of glutathione, which in turn could permit free S-adenosylmethionine to chemically methylate other

nucleophiles, such as DNA. Studies in which liver glutathione levels were depleted failed to support this hypothesis; when glutathione levels were depleted by the non-specific agent, diethyl maleate, the hydrazine - DNA methylation response was completely blocked, but when glutathione levels were depleted by specifically inhibiting the synthesis of this nucleophile with buthionine sulfoximine, hydrazine administration resulted in the usual levels of 7-methylguanine and O^6-methylguanine in liver DNA. Hence, the hepatic level of reduced glutathione does not appear to be important in this DNA methylation response to hydrazine administration.

The reproducibility of the liver DNA methylation response to hydrazine administration has been improved remarkably. The experimental conditions for this response are now firmly established, and our results have been confirmed independently by Quinter-Ruiz and coworkers (1981) for 7-methylguanine and by Dr. Peter F. Swann (personal communication) for O^6-methylguanine. Difficulty is still being experienced in achieving the same degree of reproducibility for other hepatotoxins.

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